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Sullivan 9/19/89

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Group Art Unit: 182
BOYSE et al.	Examiner: Rosen, S. 8
Serial No.: 07/119,746	
Filed: November 12, 1987	
For: ISOLATION AND PRESERVATION OF FETAL AND NEONATAL HEMATO-POIETIC STEM AND PROGENITOR CELLS OF THE BLOOD	
File No.: 6287-003	1155 Avenue of the Americas New York, New York 10036

DECLARATION UNDER 37 C.F.R. §1.132

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Hal E. Broxmeyer, do declare that:

1. I am one of the inventors named in the above-captioned application.

2. I received the degree of Bachelor of Science in Biology from Brooklyn College of the City University of New York in 1966 and the degree of Doctor of Philosophy in Cell Biology from New York University in 1973.

3. The experiments in the study described below were performed by me, my co-inventors and my collaborators, as agreed upon by the inventors.

CERTIFICATE UNDER 37 CFR 1.8(a)

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231, on 9-5-89
(Date of Deposit)

Adriane Antler 32,605
(Reg. No.)

9-5-89
(Date of Signature)

4. The purpose of the study described below was to effect the hematopoietic reconstitution of a 5 year old patient afflicted with Fanconi's anemia by the use of stem/progenitor cells from cyropreserved human umbilical cord blood. The umbilical cord blood was obtained from the patient's HLA compatible healthy sister at birth.

5. While the patient's mother was pregnant with the healthy sibling (the donor), amniotic fluid was removed and amniotic cells were cultured. Cytogenetic analysis (Auerbach et al., Blood 73:391-396, 1989; Pediatrics 76:794-800, 1988) of the cultured amniotic fluid cells showed that baseline and diepoxybutane (DEB)-induced breakage were within the normal limits, as shown in Appendix A attached hereto. These data indicate that the donor was not afflicted with Fanconi's anemia. HLA typing of amniotic fluid cells showed that the fetus was HLA identical to the patient. The HLA compatibility between the patient and the donor was confirmed at birth by HLA typing as shown in Appendix B attached hereto.

6. Immediately upon uncomplicated vaginal delivery of the donor sibling, 150 ml of umbilical cord blood was collected by gravity drainage of the delivered placenta into wide-mouthed glass bottles containing anti-coagulant, acid-citrate dextrose, and penicillin-streptomycin. Most of blood was cryopreserved by freezing the cells in a final concentration of 10% v/v dimethyl sulphoxide (DMSO) using a liquid nitrogen programmed freezer prior to placing the bags for storage in the liquid phase of liquid nitrogen.

7. The volume of blood and numbers of nucleated and hematopoietic progenitor cells collected are shown in Appendix C, attached hereto. CFU-GM was assayed using procedures described in Section 6.6.1. of the above-

identified application. BFU-E, CFU-GEMM, and CFU-GM progenitor cells were assayed using the procedures described in Section 6.6.2. of the specification. Test thaws demonstrated 79% to 90% recovery of nucleated cells, with the following recoveries of progenitor cells: 100% of day 14 CFU-GM, 63 $\pm$ 18% of BFU-E, and 70 $\pm$ 15% of CFU-GEMM.

8. The patient was administered cyclophosphamide intravenously at a dosage of 5 mg/kg/day at six, five, four, and three days prior to neonatal infusion, for a total of 20 mg/kg. One day prior to infusion, the patient was subjected to thoraco-abdominal irradiation with 500 rads and administered cyclosporin A. Approximately 300 ml of the thawed donor blood sample (containing cells, diluent and cryopreservant) was infused intravenously into the patient. Two hours after the infusion, the patient had chills, fever, and hypotension. These symptoms subsided quickly.

9. The recovery of patient blood counts is shown for up to 282 days in Appendix D, attached hereto. Reticulocytes and granulocytes began to rise by day 22 after cord blood transplantation. On day 43, blood counts showed Hb (hemoglobin) 11.3 g/dl, leucocytes 5.1 x 10^9/l, granulocytes 2.4 x 10^9/l, lymphocytes 1.9 x 10^9/l, reticulocytes 90 x 10^9/l, and platelets 31 x 10^9/l.

10. The frequency (colonies and clusters per number of cells plated) of bone marrow hematopoietic progenitor cells occurring in the recipient 1 day prior to the transplant, and 30 and 100 days post infusion of umbilical cord blood are given in Appendix E, attached hereto. Prior to the transplant, there were few or no detectable progenitor cells. At 30 days post-transplant, CFU-GM, but not BFU-E or CFU-GEMM, were detected, but by 100 days post-transplant, normal to supranormal frequencies of

these progenitors were apparent. The patient's blood cell counts have maintained in these normal ranges for at least 11 months.

11. The patient's B Rh+ red blood cells disappeared progressively and were undetectable on day 90 after transplant; 46 days after the last transfusion, 100% of red cells were of donor type (O Rh+ and remain of donor type 240 days after transplant.

12. The results of cytogenetic studies of bone marrow on day 120 post transplant, and of peripheral blood from the recipient on days 50, 64, 120 and 204 post-transplant, are shown in Appendix F, attached hereto. The chromosomal complement of the bone marrow was 46,XX. Diepoxybutane (DEB) was added to bone marrow cultures at the time of initiation and cells were harvested after 24 hours. Peripheral blood was cultured in the presence of phytohemagglutinin (PHA) for 72 hours, with DEB present in the medium for the last 48 hours of culture. The frequency of baseline and DEB-induced chromosomal breakage was analyzed using Giemsa-stained metaphase preparations; analysis of the ratio of male to female cells was also performed on quinicrine-stained slides to facilitate the identification of the Y chromosome. No male metaphases were seen in 50 quinicrine-stained cells analyzed. Chromosome breakage frequencies were 0.04 and 0.30 breaks per cell in baseline and diepoxybutane (DEB)-treated cultures, respectively. Through day 120 post-transplant, more than 50% of the metaphases seen in cytogenetic preparations from PHA-stimulated peripheral blood cultures were of host origin, as demonstrated by the presence of a Y chromosome. These cells, of lymphoid origin, exhibited highly elevated baseline chromosomal breakage, and severe radiation damage in the form

of multiple dicentrics, rings, and chromosomal fragments. These male cells were also hypersensitive to the clastogenic effect of DEB, showing multiple chromatid breaks and exchanges typical of Fanconi's anemia. Female cells did not exhibit elevated levels of chromosomal breakage. By day 204 (6 1/2 months) post-transplant, the majority of peripheral blood lymphocytes were of donor origin.

13. DNA samples from the parents, donor, HLA unmathed sibling, and recipient (pre- and post-transplant) were analyzed by Southern Blot hybridization. The results are shown in Appendix G, attached hereto. DNA from the peripheral blood of the father (lane 1), mother (lane 2), patients pre-transplant (lane 3), donor (lane 4), HLA non-identical sister (lane 5), patient post-transplant day 50 (lane 6), day 64 (lane 7), day 120 (lane 8) was digested with the restriction enzyme Taq I, (New England Biolabs), separated by gel electrophoresis, transferred to a nylon membrane, hybridized, and washed. The probe used was CRI-pS232 (DXS278) (Collaborative Research, Boston, MA), which recognizes a complex set of fragments at a highly polymorphic locus on the X chromosome as well as a locus on the Y chromosome. On the right side of the figure the heavy arrows denote X-specific bands from the donor while the lighter arrows denote X-specific bands from the recipient. Y-specific bands are of 4.9 and 3.8 kb. A constant band of 3.0 kb is seen in all individuals. The DNA from the patient post-transplant is primarily of donor origin.

14. Evaluation of the results of the above studies indicate that the cord blood of the HLA-matched sibling, after cryopreservation and thawing, reconstituted the Fanconi's anemia patent. Virtually complete occupation of the male patient's myeloid system by female sibling donor

cells was demonstrated by cytogenetics, lack of undue chromosomal fragility, ABO typing and DNA matching. At present, the peripheral blood is a chimera with some of the recipient's radiation damaged lymphoid cells remaining in the circulation, but the treat has been towards elimination of these damaged cells.

15. I therefore conclude that cryopreserved human umbilical cord blood from a single individual may be used as a source of stem/progenitor cells for hematopoietic reconstitution.

16. I declare that all statements made in this declaration are of my own knowledge and are true and that all statements made on information and belief are believed to be true and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of the Title 18 of the United States Codes and that such willful false statements may jeopardize the validity of the application or patent issuing thereon.

Dated: Aug. 31, 1989

Hal E. Broxmeyer

Dr. Hal E. Broxmeyer

APPENDIX A

CYTOGENETIC ANALYSIS

	Mean Chromosome Breaks/Cell	
	Spontaneous	DEB Induced (0.1 g/ml)
Patient PBL	0.18	10.6[a]
Donor Amniotic fluid	0	0.03[b]
Donor Cord blood	-	0.02[a]
Control range	0-0.02	0-0.10

[a]PHA-stimulated lymphocytes from patient peripheral blood (PBL) and cord blood were exposed in culture to 0.1 μg/ml DEB.

[b]Cultured amniotic fluid cells were exposed to 0.01 μg/ml DEB.

APPENDIX B

HLA TYPING

	First Haplotype	Second haplotype	ABO
Father	A3 B18 DR6	A29 B44 DR3	
Mother	A1 B8 DR3	A11 B39 DR3	
Sister	A1 B8 DR3	A3 B18 DR6	
Patient	A1 B8 DR3	A29 B44 DR3	B+
Donor (Sister)	A1 B8 DR3	A29 B44 DR3	O+

APPENDIX C

NUCLEATED CELLULARITY AND NUMBERS OF HEMATOPOIETIC PROGENITOR CELLS IN BLOOD FROM UMBILICAL CORD AND PLACENTA

Parameters Evaluated	Cord	Placental	Total
Volume of Blood Collected (ml)	130	30	160
Nucleated Cells x 10^{-9}	1.05	0.14	1.19
Hematopoietic Progenitor Cells x 10^{-5}			
a) Agar Culture			
Day 14 CFU-GM (colonies)	1.4	0.12	1.52
Day 14 CFU-GM (colonies & clusters)	2.2	0.26	2.46
b) Methyl Cellulose Cultures (Colonies)			
Day 14 CFU-GM	1.3	0.26	1.56
BFU-E-2	3.7	0.25	3.95
BFU-E-1	3.4	0.20	3.60
CFU-GEMM	0.36	0.03	0.39

APPENDIX D

Blood Counts After Cord Blood Transplanation

Days	Hemoglobin	Leucocytes	Granulocytes	Lymphocytes	Platelets	Reticulocyte
-20	6.8	3.1	0.25	2.8	18	10
0	9.7	0.8	0	0.8	120	0
8	10.9	0.4	0	0.4	80	0
15	11.6	0.4	0	0.4	39	0
22	7.8	0.9	0.3	0.6	50	5
29	8.5	1.0	0.3	0.5	105	17
36	9.4	1.7	0.6	0.5	55	36
43	11.3	5.1	2.4	1.9	31	90
50	8.9	3.4	1.5	0.7	62	162
57	8.9	5.6	3.2	1.0	174	63
90	11.3	5.1	4.0	1.1	296	50
120	13	3.9	2.3	1.1	265	40
160	12	3.7	1.4	1.6	293	45
240	12.3	5.2	2.7	1.6	354	50
282	12.2	4.8	2.3	1.2	315	--

Hemoglobin (Hb) is expressed in g per dl, leucocytes, granulocytes, lymphocytes platelets, and reticulocytes are expressed as numbers in 10^9 per liter.

APPENDIX E

NUMBERS OF HEMATOPOIETIC PROGENITOR CELLS IN RECEIPIENT BONE MARROW IMMEDIATELY PRIOR TO CONDITIONING AND 30 AND 100 DAYS POST-TRANSPLANT

Progenitors In Recipient Bone Marrow Per 10^5 cells plated	Unseparated	Low Density (5.077gm/ml)
1) Pre-Transplant Methylcellulose Culture		
Day 14 CFU-GM (colonies)	ND	1[b]
Day 14 CFU-GM (colonies) clusters)	ND	8
BFU-E-1	ND	0
CFU-GEMM	ND	0
2) Day 30 Post-Transplant Methylcellulose Culture		
Day 14 CFU-GM	ND	9
BFU-E-1	ND	0
CFU-GEMM	ND	0
3) Day 100 Post-Transplant		
a) Agar Cultures		
Day 7 CFU-GM (colonies)	22 ± 2	140 ± 12
Day 7 CFU-GM (colonies & clusters)	118 ± 16	416 ± 10
Day 14 CFU-GM (colonies)	36 ± 4	156 ± 8
Day 14 CFU-GM (colonies & clusters)	152 ± 4	228 ± 12
b) Methylcellulose Culture		
Day 14 CFU-GM (colonies	324 ± 16	416 ± 25
BFU-E-2	77 ± 2	134 ± 6
BFU-E-1	96 ± 8	132 ± 10
CFU-GEMM	4 ±	9 ± 1

[a]Results are expressed as mean ± 1 S.E.M.

[b]These were microclusters, less than 20 cells/cluster

ND, not done.

APPENDIX F

CYTOGENETIC ANALYSIS OF BONE MARROW AND PERIPHERAL BLOOD FROM RECIPIENT OF UMBILICAL CORD BLOOD TRANSPLANT

Tissue	No. Days Post transplant	% Female Cells In Recipient[a]		Mean Chromosome Breaks/Cell[b]	
		Baseline	DEB-treated[c]	Baseline	DEB-treated[c]
Bone Marrow	120	100	100	0.04	0.30
Peripheral Blood	50	30	52	0.7	4.0
Peripheral Blood	64	8	32	1.3	5.0
Peripheral Blood	120	12	32	1.9	6.4
Peripheral Blood	204	64	86	0.30	0.49

[a] Analysis performed on quinicrine-stained metaphase preparations.

[b] Analysis performed on Giemsa-stained metaphase preparations.

[c] Final concentration of DEB in the medium was 0.1 mg/ml.

APPENDIX G